Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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Induction of complete remissions of ALL by chimeric antigen receptor-expressing T cells

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Methods

General laboratory statement: Research sample processing, freezing, and laboratory analyses were performed in the Translational and Correlative Studies Laboratory at the University of Pennsylvania, which operates under principles of Good Laboratory Practice with established SOPs and/or protocols for sample receipt, processing, freezing, and analysis. Assay performance and data reporting conforms with MIATA guidelines.[1]

Sample draws and processing: Samples (peripheral blood, bone marrow) were collected in lavender top (K_2EDTA,) or red top (no additive) vacutainer tubes (Becton Dickinson). Lavender top tubes were delivered to the laboratory within 2 hours of draw, or shipped overnight at room temperature in insulated containers essentially as described[2] prior to processing. Samples were processed within 30 minutes of receipt according to established laboratory SOP. Peripheral blood and marrow mononuclear cells were purified, processed, and stored in liquid nitrogen as described.[3] Red top tubes were processed within 2 hours of draw including coagulation time; serum isolated by centrifugation, aliquoted in single use 100 μL aliquots and stored at -80^{o} C. CSF was delivered to the laboratory within 30 minutes of aspiration and cells in CSF were collected by centrifugation of CSF fluid and processed for DNA and flow cytometry.

Q-PCR analysis: Whole-blood or marrow samples were collected in lavender top (K_2EDTA) BD vacutainer tubes (Becton Dickinson). Genomic DNA was isolated directly from whole-blood and Q-PCR analysis on genomic DNA samples was performed in bulk using ABI Taqman technology and a validated assay to detect the integrated CD19 CAR transgene sequence as described[3]

using 200 ng genomic DNA per time-point for peripheral blood and marrow samples, and 18-21.7 ng genomic DNA per time-point for CSF samples. To determine copy number per unit DNA, an 8-point standard curve was generated consisting of 5 to 10^6 copies CTL019 lentivirus plasmid spiked into 100 ng non-transduced control genomic DNA. Each data-point (sample, standard curve) was evaluated in triplicate with a positive Ct value in 3/3 replicates with % CV less than 0.95% for all quantifiable values. A parallel amplification reaction to control for the quality of interrogated DNA was performed using 20 ng input genomic DNA from peripheral blood and marrow (2-4.3 ng for CSF samples), and a primer/probe combination specific for non-transcribed genomic sequence upstream of the CDKN1A gene as described[3]. These amplification reactions generated a correction factor (CF) to correct for calculated versus actual DNA input. Copies of transgene per microgram DNA were calculated according to the formula: copies calculated from CTL019 standard curve per input DNA (ng) x CF x 1000 ng. Accuracy of this assay was determined by the ability to quantify marking of the infused cell product by Q-PCR. These blinded determinations generated Q-PCR and flow marking values of 11.1% and 11.6%, respectively, for the CHOP-100 and 20.0% and 14.4%, respectively, marking for the CHOP-101 infusion products.

Soluble factor analysis: Whole blood was collected in red top (no additive) BD vacutainer tubes (Becton Dickinson), processed to obtain serum using established laboratory SOP, aliquoted for single use and stored at -80^{o}C. Quantification of soluble cytokine factors was performed using Luminex bead array technology and kits purchased from Life technologies (Invitrogen). Assays were performed as per the manufacturer protocol with a 9 point standard curve generated using a 3-fold dilution series. The 2 external standard points were evaluated in duplicate and

the 5 internal standards in singlicate; all samples were evaluated in duplicate at 1:2 dilution; calculated % CV for the duplicate measures were less than 15%. Data were acquired on a FlexMAP-3D by percent and analyzed using XPonent 4.0 software and 5-parameter logistic regression analysis. Standard curve quantification ranges were determined by the 80-120% (observed/expected value) range. Reported values included those within the standard curve range and those calculated by the logistic regression analysis.

Antibody reagents: The following antibodies were used for these studies: MDA-CAR,[4] a murine antibody to CD19 CAR conjugated to Alexa647, was a generous gift of Drs. Bipulendu Jena and Laurence Cooper (MD Anderson Cancer Center). Antibodies for multi-parametric immunophenotyping: T cell detection panels: anti-CD3-FITC, anti-CD8-PE, anti-CD14-PE-Cy7, anti-CD16-PE-Cy7, anti-CD19-PE-Cy7 anti-CD16-PE-Cy7. B cell detection panels: anti- CD20-FITC, anti-CD45-PE, anti-CD45-APC, anti-CD19-PE-Cy7, anti-CD19-PE, anti-CD34-PCP-e710 and anti CD34-APC were procured from e-Biosciences.

Multi-parameter flow cytometry: Cells were evaluated by flow cytometry directly after Ficoll-Paque processing, with the exception of the CHOP-101 baseline sample which was evaluated immediately after thaw of a cryopreserved sample. Multi-parametric immunophenotyping for peripheral blood and marrow samples was performed using approximately 0.2-0.5 $x10^6$ total cells per condition (depending on cell yield in samples), and for CSF samples using trace amounts of cells collected following centrifugation of CSF fluid, and using fluorescence minus one (FMO) stains as described in the text. Cells were stained in 100 μL PBS for 30 minutes on ice using antibody and reagent concentrations recommended by the manufacturer, washed,

and resuspended in 0.5% paraformaldehyde and acquired using an Accuri C6 cytometer equipped with a Blue (488) and Red (633 nm) laser. Accuri files were exported in FCS file format and analyzed using FlowJo software (Version 9.5.3, Treestar). Compensation values were established using single antibody stains and BD compensation beads (Becton Dickinson) and were calculated by the software. The gating strategy for T cells was as follows: Live cells (FSC/SSC) > dump channel (CD14+CD16+CD19-PECy7) vs CD3+ > CD3+. The general gating strategy for B cells was as follows: Live cells (FSC/SSC) > SSC low events > CD19+. More gating details for the CHOP-100 and CHOP-101 samples are described in the individual Figures.

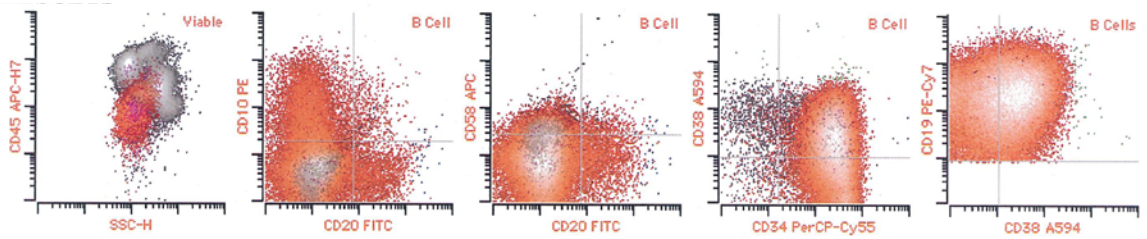
Molecular MRD analysis: Molecular MRD analysis was performed by Adaptive Biotechnologies (Seattle, WA) and high-throughput next-generation sequencing of the BCR IGH CDR3 region using the Illumina HiSeq/MiSeq platform-based immunoSEQ assay.[5] For these analyses, 701-6,000 ng (approximately 111,000- 950,000 genome equivalents) of genomic DNA isolated from whole blood or marrow samples obtained from patients were subjected to combined multiplex PCR and sequencing followed by algorithmic analyses to quantify individual IGH CDR3 sequences in samples. Parallel amplifications and sequencing of the TCRB CDR3 region[6] in each sample were done to assess quality of DNA samples. For each patient, the IGH CDR3 nucleotide sequences assayed from samples of different time points were aligned using EMBL-EBI multiple sequence alignment tool.[7,8] The dominant clone from the earliest time-point sample was bioinformatically tracked across the assayed IGH CDR3 sequences in the following time-point samples to identify presence of sequences with 95% or greater pair-wise sequence identity. The total sequencing reads for those sequences similar to the dominant clone are reported for each time-point.

Discussion

Tocilizumab (anti-IL6) has promise for glucocorticoid resistant GVHD, and our results are consistent with these data. Further, it is interesting to note that in CHOP 100, the CRS manifesting as high fever, hypotension and multi-organ failure was resistant to the high doses of glucocorticoids administered over the previous 2 days before cytokine directed therapy. Finally, in CHOP-100 the biphasic changes in IL-1β, IL-1RA and IL-2 shown in figure 1B may have been related to cytokine-directed therapy with etanercept and tocilizumab.

The induction of remission in a patient refractory to blinatumomab therapy further highlights the potency of CTL019 cells. The high efficiency of trafficking of CAR T cells to the CNS is encouraging as a mechanism for surveillance to prevent relapse in a sanctuary site such as the CNS, and supports the testing of CAR T-cell-directed therapies for CNS lymphomas and primary CNS malignancies. Neither patient has experienced cognitive effects that might be ascribed to the trafficking of T cells to the CNS.

CHOP-101 Baseline



CHOP-101 Day +23

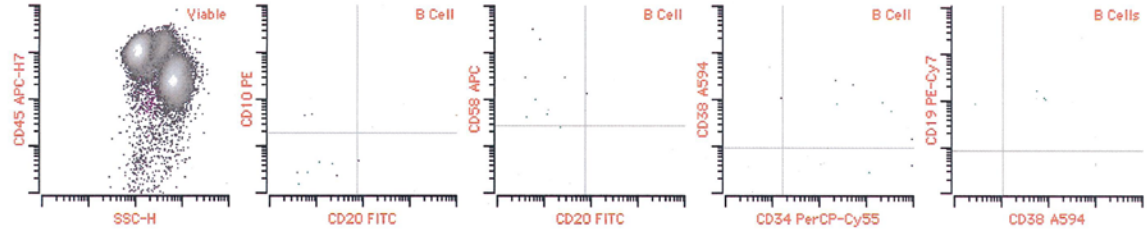


Figure S1. Induction of remission in bone marrow in CHOP-101 on day +23 after CTL019 infusion. Clinical immunophenotyping report for CHOP-101 at baseline (**Top panel**) and at day +23 (**Bottom panel**). Cells were stained for CD10, CD19, CD20, CD34, CD38 and CD58. Flow cytometry was done after lysis of the red blood cells. The report on day +23 stated "reveals that the white blood cells consist of 42.0 % lymphocytes, 6.0 % monocytes, 50.3 % myeloid forms, 0.17 % myeloid blasts and no viable lymphoid progenitors. There was no convincing immunophenotypic evidence of residual precursor B cell lymphoblastic leukemia/lymphoma by flow cytometry. Essentially no viable B cells were identified." MRD was performed at the CAP and CLIA certified Children's Oncology Group (COG) Western Immunophenotyping Reference Laboratory at the University of Washington, Seattle WA.

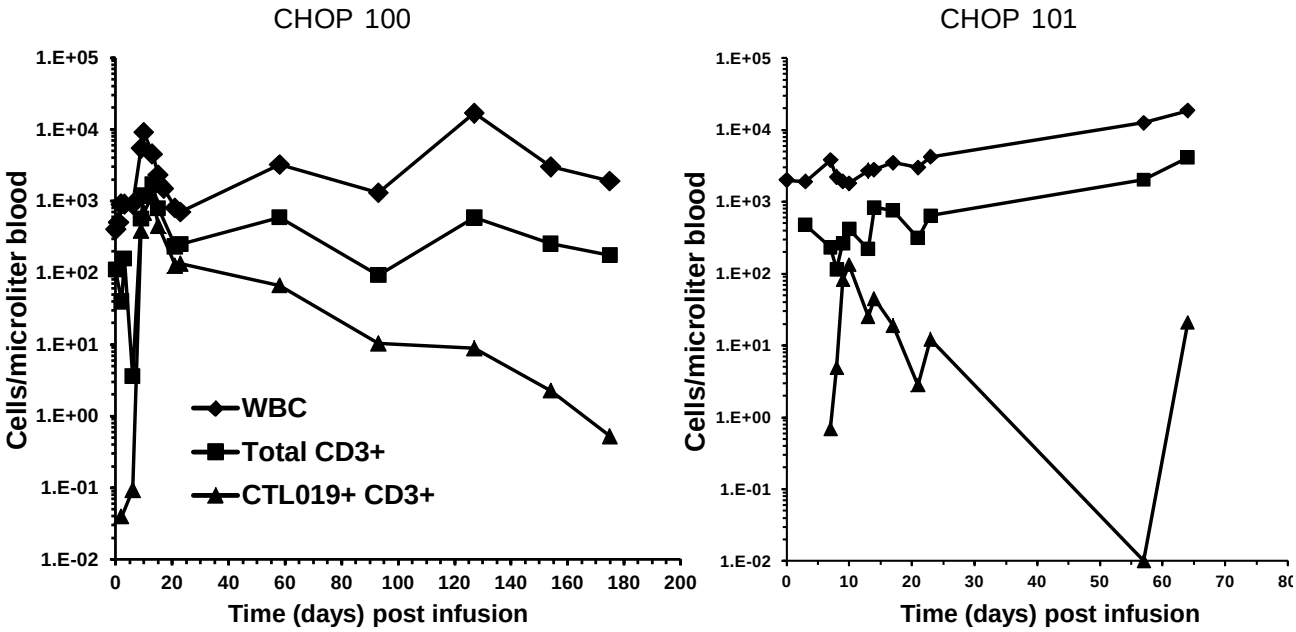


Figure S2. In vivo expansion and persistence of CTL019 cells in blood. The number of white blood cells (WBC), CD3+ T cells, and CTL019 cells in blood is shown for CHOP-100 and CHOP-101. Cell numbers are shown on a semi-logarithmic plot. Cells/microliter blood are calculated from the % CD3+ and % CD3+/CTL019 values obtained from flow cytometry and the WBC values obtained from clinical analyses.

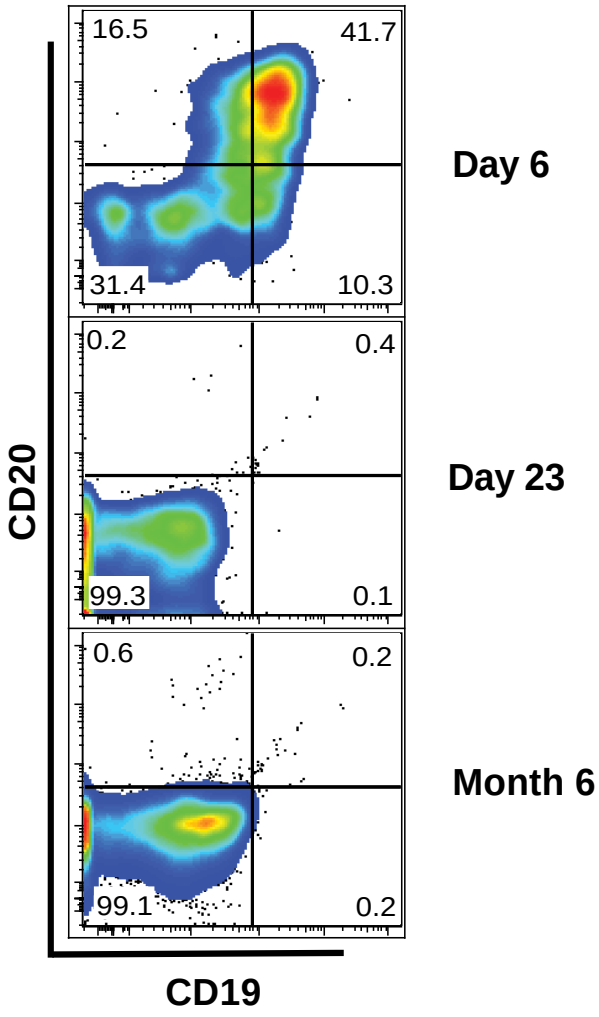


Figure S3A. Persistent B cell aplasia in CHOP-100. The top panel shows a predominant population of leukemic blast cells in bone marrow aspirated from CHOP-100 expressing CD19 and CD20 on day +6. This population is absent at day +23 and 6 months.

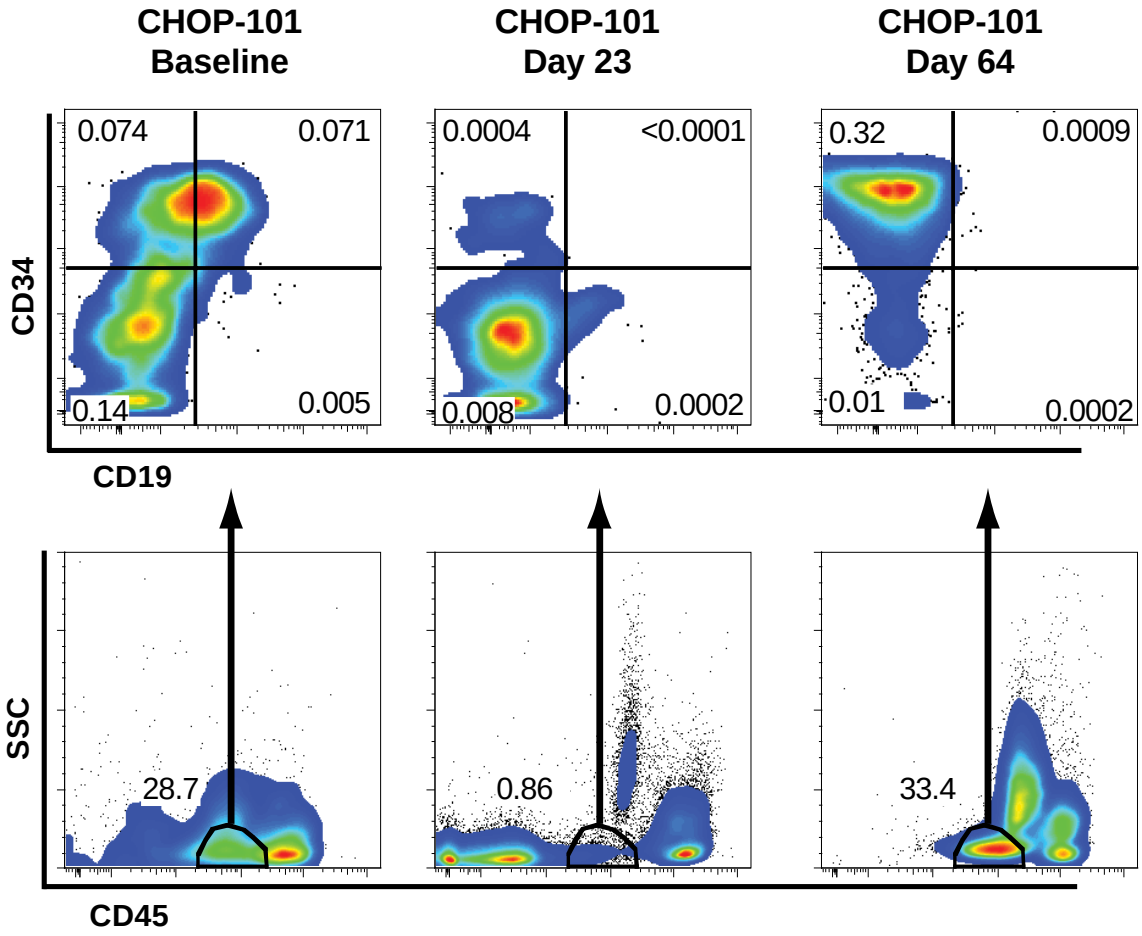


Figure S3B. B cell aplasia and emergence of CD19 escape variant cells in CHOP-101. Flow cytometric analysis of bone marrow aspirates from CHOP-101 stained with anti-CD45, CD34 and CD19. In the bottom row, side scatter and the CD45 dim positive cells were used to identify leukemic cells that express variable amounts of CD34 and CD19 at baseline. Only CD19 negative blasts were detected on day 64. Numerical values in the top panel represent the fraction of the total leukocytes represented in each quadrant. Numerical values in the lower panel represent the percentage from the total leukocytes represented in the CD45dim/SS low gate

Supplemental References

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